Juxta-articular joint-capsule mineralization in CD73 deficient mice: Similarities to patients with *NT5E* mutations

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Arterial calcification due to CD73 deficiency (ACDC), an autosomal recessive disorder, manifests with extensive mineralization of the lower-extremity arteries as well as of hand and foot joint-capsules. This disease is caused by mutations in the *NT5E* gene which encodes CD73, a membrane-bound ecto-5′-nucleotidase hydrolyzing 5′-AMP into adenosine and P_i. To gain insight into the pathophysiologic details of ACDC, we have characterized a $Nt5e^{-/-}$ knock out mouse ($Nt5e^{tm1Jgsc}$) deficient in CD73. These mice, when maintained on appropriate strain background, demonstrated stiffening of the joints and micro CT revealed distinct changes in the thoracic skeletal structure with evidence of mineralization at the costochondral junctions. Mineralization was also noted in the juxta-articular spaces of the lower extremities as well as of ligaments and capsules adjacent to the bony structures. No evidence of vascular mineralization was noted either by CT or by microdissection of arteries in the thoracic area or in lower extremities. The $Nt5e^{-/-}$ mutant mice demonstrated significantly increased P_i levels in the serum and significantly reduced PP_i concentration in the heparinized plasma, resulting in markedly increased P_i/PP_i ratio, thus creating a pro-mineralization environment. In conclusion, the $Nt5e^{-/-}$ targeted mutant mice recapitulate some, but not all, features of ACDC and serve as a model system to study pharmacologic interventions for ectopic mineralization. Collectively, this mouse model deficient in CD73, with other targeted mutant mice with vascular mineralization, attests to the presence of a complex pro-mineralization/anti-mineralization network that under physiologic homeostatic conditions prevents ectopic tissue mineralization.

Introduction

CD73 is a membrane-bound ecto-5′-nucleotidase that catalyzes hydrolysis of extracellular nucleoside monophosphates into nucleoside intermediates.[1,2] Specifically, CD73 converts 5′-AMP into adenosine and inorganic phosphate (Pi). The newly generated adenosine, acting on 4 G-protein receptors, plays critical physiological roles and can also be involved in pathological processes.[3] At the same time, P_i can serve as a pro-mineralization factor, particularly in the context of increased inorganic phosphate/pyrophosphate (P_i/PP_i) ratio.[4]

In 2004, 3 research groups independently generated knock out mice by targeted disruption of the mouse *Nt5e*, the gene encoding CD73.[5-7] These mice were subsequently characterized to show altered thromboregulation and augmented vascular inflammatory response, lack of CD73 was shown to promote arteriogenesis, and the $Nt5e^{-/-}$ mice manifested with fulminant vascular leakage during hypoxia. In 2011, St. Hilare et al. reported on 3 families with 9 affected individuals with mutations in the *NT5E* gene which encodes human CD73 – this disease is known as arterial calcification due to CD73 deficiency (ACDC).[8] These patients, as originally described about 100 y ago,[9,10] manifested with extensive calcification of the lower-extremity arteries as well as hand and foot joint-capsules. In particular, contrast-enhanced magnetic-resonance angiography revealed extensive occlusion of the iliac, femoropopliteal and tibial arteries, and X-ray findings showed heavy calcification with areas of arteriomegaly. Interestingly, no vascular calcification was detected by chest X-rays above the diaphragm. Radiography also revealed juxta-articular joint-capsule calcifications in the digits, wrists, ankles and feet. These 3 families were found to have loss-of-function mutations in the *NT5E* gene, and cultured fibroblasts from affected members from one of the families showed markedly reduced expression of NT5E mRNA, CD73 protein,

and enzymatic activity.[8,11] These cells were also shown to have increased alkaline phosphatase levels and accumulation of calcium phosphate crystals.

With our long term interest in heritable ectopic mineralization disorders affecting primarily the skin and arterial blood vessels,[12] we have now characterized a mutant $Nt5e^{-/-}$ mouse deficient in CD73, and we demonstrate that these mice recapitulate some, but not all, clinical features noted in patients with *NT5E* mutations.

Results

Generation of *Nt5e^tm1jgsc* mice

CD73-deficient mice, generated by targeted inactivation of the *Nt5e* gene, as described previously,[5] were obtained from the National Institutes of Health (courtesy of Dr. Jurgen Schnermann). These mutant mice, *Nt5e^tm1Jgsc*, are referred from here on as $Nt5e^{-/-}$ mice. These mice failed to demonstrate *Nt5e* mRNA sequences beyond the targeted 2nd and 3rd exon, and they lacked CD73 by immunostaining of kidneys, the site depicting the presence of the protein in wild-type mice. The breeding pairs, which were on the 129SvEv strain background, were used to generate homozygous, heterozygous, and wild-type mice with respect to the mutant *Nt5e* allele. These mice were placed on a specific diet, so-called "acceleration diet" rich in phosphate and low in magnesium, which we have previously shown to accelerate the mineralization in *Abcc6^tm1Jfk* and *Enpp1^asj* mutant mice, animal models of pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI), two ectopic heritable mineralization disorders, respectively.[13-15] The animals were sacrificed at different time points up to 11 months of age and examined histologically for systemic mineralization. Initially, all mice, irrespective of the *Nt5e* gene, demonstrated, extensive mineralization in soft connective tissues, including the dermal sheath of vibrissae and arterial blood vessels, similar to previous demonstrations in a number of mouse strains serving as models of PXE. Specifically, we have previously shown that 4 mouse strains with ectopic mineralization, *viz.*, 129S1/SvImJ, C3H/HeJ, DBA/2J, and KK/HlJ, harbor a single nucleotide polymorphism (SNP; rs32756904) in the *Abcc6* gene (**Fig. 1**).[16] This nucleotide variant elicits aberrant splicing of the *Abcc6* mRNA, causing deletion of 5 nucleotides at the end of exon 14 and resulting in a frame shift and premature termination codon of translation.[17,18] As a consequence ABCC6 protein levels in the liver are markedly reduced, associated with ectopic mineralization of peripheral connective tissues. Examination of the *Abcc6* genomic sequence in the $Nt5e^{-/-}$ mice revealed the presence of this SNP (**Fig. 1**), and consequently, this mutation is likely to explain the mineralization phenotype in these mice independent of the *Nt5e* mutation status.

Phenotypic characterization of Nt5e^−/− mice

To separate the *Nt5e* locus in chromosome 9 from the *Abcc6* mutant allele in chromosome 7, the *Nt5e* mutant mice were crossed onto C57BL/6J background by 3 back crosses, which eliminated the mutant allele of the *Abcc6* gene (**Fig. 1**). These mice were subsequently intercrossed to generate homozygous, heterozygous, and wild-type mice with respect to the *Nt5e* mutant allele. These mice were then placed on the acceleration diet.

To explore the potential of mineralization, complete necropsies of the $Nt5e^{-/-}$ mice were performed at 11–16 months of age.[19] These mice, together with heterozygous and wild-type mice, developed mineralization in the kidneys, a finding that we previously demonstrated in mice kept on this particular "acceleration diet."[20] However, no evidence of soft tissue calcification, particularly in arteries, was noted in $Nt5e^{-/-}$ mice. It should be noted, however, that the complete necropsy protocol utilized does not allow detection of mineral deposits on cartilage or on soft tissues adjacent to long bones due to decalcification of skeletal tissues.

Since ACDC patients develop mineralization of cartilage and
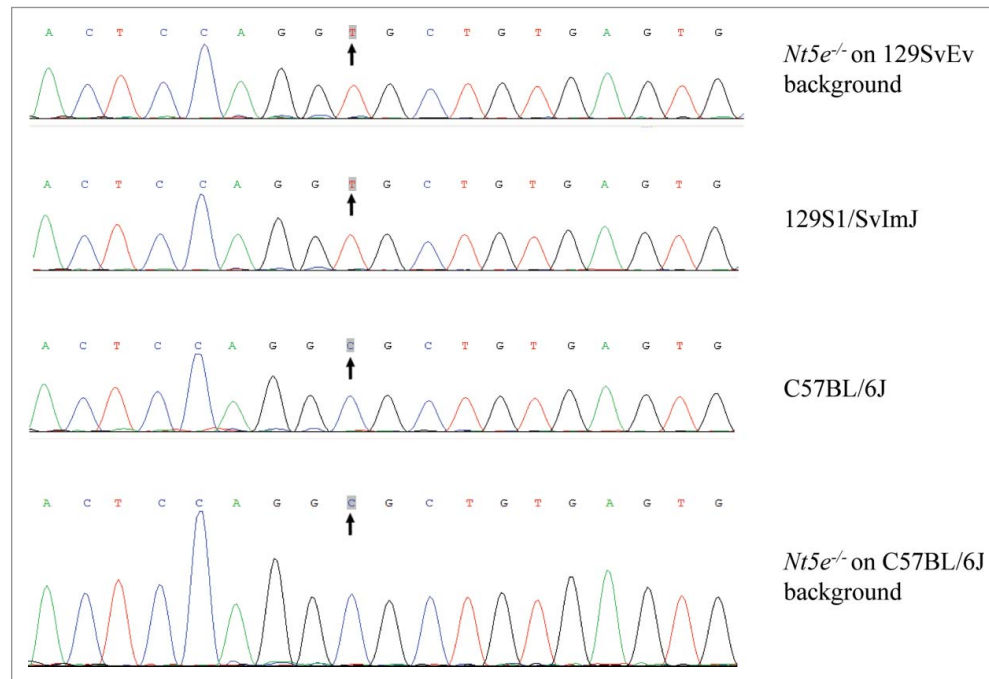

Figure 1. Genomic sequences of a SNP (rs32756904) in the *Abcc6* gene of mice on different strain backgrounds. The original $Nt5e^{-/-}$ mouse was developed on 129SvEv background (top line) which harbors a homozygous T nucleotide similar to 129S1/SvImJ (2nd row from top). The T allele is associated with ectopic mineralization. The *Nt5e* allele was separated from the mutant *Abcc6* allele by crossing the mice on to C57BL/6J strain background which is homozygous for the C allele of the SNP (lower rows).
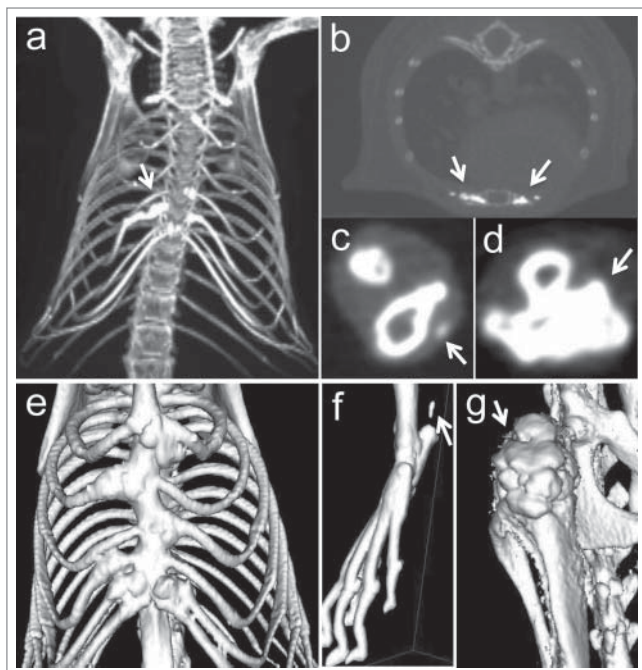
Figure 2. Representative images of micro CT scans of $Nt5e^{-/-}$ mice of 11–16 months of age. Note extensive ectopic mineralization of the sternocostal junctions (**A, B**), juxta-articular tissues in lower extremities (**C**), and knee (**D**) (arrows). Three-D renderings reveal skeletal deformities due to ectopic mineralization of thorax (**E**), foot (**F**), and knee (**G**). Note mineralization of the Achilles tendon in (**F**) (arrow).

examination of the extremities by the micro CT scan revealed evidence suggestive of ectopic mineralization of ligaments and capsules adjacent to the bone structures (**Fig. 2**). No evidence of aberrant mineralization was noted in heterozygous or wild-type littermates.

With particular attention to the vascular mineralization in ACDC patients, we performed micro-dissection of saphenous, medial tarsal, medial marginal, and lateral marginal arteries as well as internal mammary and intercostal arteries in $Nt5e^{-/-}$ mice. Histopathologic examination by Hematoxylin-Eosin and Alizarin Red stains failed to provide evidence of vascular mineralization.

Finally, clinical assays were performed in the blood of the $Nt5e^{-/-}$ mutant mice to determine the serum concentration of calcium and inorganic phosphate (P_i) and the plasma inorganic pyrophosphate (PP_i) (**Table 1**). No difference in the serum calcium concentration was noted between the 3 groups of mice. However, the P_i levels in the serum of $Nt5e^{-/-}$ mice were significantly increased, by 43% on the average, as compared to wild-type mice ($p < 0.01$). At the same time, the plasma PP_i concentration was significantly reduced, by ~50%, in the knock out mice as compared to their wild-type littermates. Consequently the P_i/PP_i ratio was increased by 2.27-fold in the knockout mice compared to the corresponding controls. The heterozygous $Nt5e^{+/-}$ mice showed P_i and PP_i values intermediate between the corresponding homozygous and wild-type mice, but they were not statistically different from the wild-type mice due to individual variations. No difference was noted in alkaline phosphatase activity in serum.

juxta-articular joint-capsules as well as in arterial blood vessels of the lower extremities,[8] potential development of ectopic mineralization on these sites was monitored in live animals with micro computed tomography (CT) scan at various time points. CT imaging of $Nt5e^{-/-}$ mice revealed consistently distinct changes in the thoracic skeletal structures with evidence of juxta-articular mineralization of the costochondral junctions (**Fig. 2**). Evidence of mineralization was also noted in the juxta-articular spaces of the lower extremities most notably at the level of the patella and the distal insertion of the fibula and tibia. Mineralization of the Achilles tendon was also observed. In contrast, no juxta-articular mineralization of the upper extremities was found. Careful

Discussion

A number of heritable disorders have been shown to affect the peripheral connective tissues by ectopic mineralization. The prototype for such conditions is PXE, a multisystem mineralization disorder affecting the skin, the eyes, and the cardiovascular system.[21] This disease, caused by a mutations in the *ABCC6* gene, characteristically shows late onset and slow progression of the pathological mineralization processes. In contrast, GACI, caused by mutations in the *ENNP1* gene, is frequently diagnosed by pre- or perinatal ultrasound and the affected individuals, born with extensive mineralization of arterial blood vessels, die in

Table 1. Blood parameters in *Nt5e* wild type, as well as heterozygous and homozygous mutant mice

Nt5e genotype*	Serum/plasma concentration (mean ± S.E.)					
	Ca^{2+} (mg/dl)	P$_i$ (mg/dl)	Ca/P$_i$ ratio	PP$_i$ (μM)	P$_i$/PP$_i$ ratio	Alp activity (U/L)
WT	10.20 ± 0.27	9.89 ± 0.67	1.05 ± 0.04	2.47 ± 0.39	269.60 ± 48.03	90.97 ± 10.00
Het	9.72 ± 0.11	11.83 ± 1.12	0.87 ± 0.07	2.11 ± 0.52	493.21 ± 110.93	114.76 ± 17.96
KO[+]	10.05 ± 0.13	14.19 ± 0.82[#]	0.73 ± 0.04[#]	1.30 ± 0.16[#]	672.38 ± 111.13[*]	95.76 ± 9.67

*WT, wild type; Het, heterozygous; KO, knock out.
[+]Statistical significance in comparison to wild type mice is indicated: * $p < 0.05$; # $p < 0.01$ (n = 5–12). Calcium, P$_i$ and Alp activity were measured in serum samples, and PP$_i$ was measured in heparinized plasma samples.
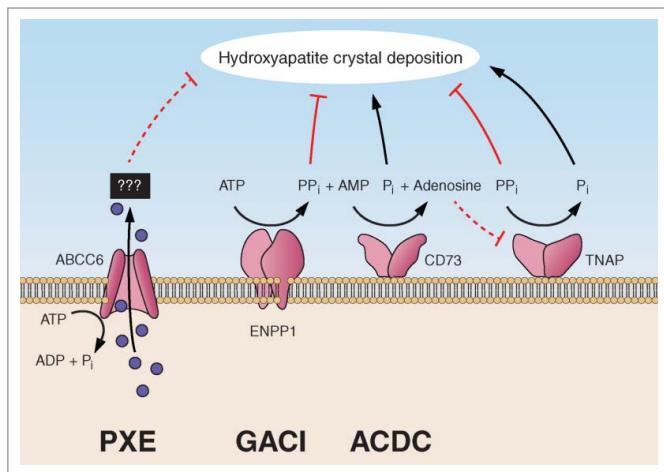Alp, alkaline phosphatase.

Figure 3. Schematic presentation of factors contributing to the pro-mineralization/anti-mineralization network preventing ectopic mineralization under physiologic conditions. Genetic defects in the genes encoding ABCC6, ENPP1 and CD73 result in ectopic mineralization in pseudoxanthoma elasticum (PXE), generalized arterial calcification of infancy (GACI) and arterial calcification due to CD73 deficiency (ACDC), respectively. (Modified from reference 12).

most cases from cardiovascular complications during the first year of life.[22] ACDC, arterial calcification due to CD73 deficiency, has been reported in elderly patients with calcification of arteries in the lower extremities, associated with mineralization of the juxta-articular joint-capsules.[8] These patients harbor loss-of-function mutations in the *NT5E* gene which encodes CD73, an ecto-5′-nucleotidase that catalyzes the hydrolysis of 5′-AMP into adenosine and P_i. It has been proposed that under physiological homeostasis adenosine inhibits the activity of tissue non-specific alkaline phosphatase (TNAP), an enzyme that converts PP_i to P_i.[8,11] In CD73 deficiency the tissue levels of adenosine are reduced, lessening the inhibition of TNAP, and consequently the generation of P_i from PP_i is accelerated, and the ratio of P_i/PP_i increases creating a pro-mineralization environment (**Fig. 3**).

A number of animals, particularly targeted or spontaneous mutant mice, have served as models of the ectopic mineralization disorders, and they have been extremely helpful in deciphering the pathophysiologic details of many of these conditions.[12,23] For example, generation of the *Abcc6*^{−/−} mice as a model for PXE has greatly facilitated the understanding of the pathomechanistic features of this complex disorder.[24,25] Similarly, generation of targeted mutant *Enpp1*^{−/−} mice or identification of spontaneous *Enpp1* mutant mice, such as asj and ttw, have provided novel insight into the mineralization processes in GACI. These mouse models now serve as a platform to test novel treatment modalities for these, currently intractable diseases.[15,26,27]

Several targeted mutant mice have been developed by disruption of the *Nt5e* gene resulting in CD73 deficiency, but very little attention has been paid so far on the mineralization aspects of these mice.[5-7] In this study, we have characterized a CD73 deficient mouse which was generated by targeted interruption of the *Nt5e* gene with complete absence of the CD73 protein, a true

null mutation.[5] Combination of clinical phenotypes and CT imaging studies indicated extensive mineralization of the juxta-articular joint-capsules in the thoracic area with extensive skeletal abnormalities. In addition, extensive mineralization of the joint capsules, particularly in the lower extremities, was evident. Evidence of ectopic calcification was also noted in soft tissues adjacent to the bone, including the Achilles tendon. Thus, this CD73 deficient mouse model, particularly when placed on an "acceleration diet" which facilitates the mineralization processes, recapitulates features noted in patients with ACDC. However, CT scan or extensive histopathologic evaluation of the soft tissues in the lower legs or in the thoracic area failed to reveal mineralization in the arterial blood vessels, a prominent feature in patients with ACDC.[8] The reasons for the lack of vascular mineralization in the mice are currently unknown, but could involve the genetic background or epigenetic factors. The former point was clearly demonstrated by changing the strain background to remove the *Abcc6* mutation in the original 129SvEv strain to C57BL/6J. Furthermore, the physiological diversity of the cardiovascular system, including the arterial blood vessels, is highly complex and includes positional identities determined by *Hox* expression in vascular smooth muscle cells and endothelial cells.[28] Presumably, such factors could differ between humans and mice. In addition, a number of genetic modifiers could differentially influence the expression of the phenotype in human and mice in diseases which are thought to be single gene disorders, yet demonstrate considerable phenotypic variability.[29] Nevertheless, this mouse model can be used to study the pathomechanistic features and metabolic pathways potentially involved in the development of mineralization in ACDC.

As indicated above, the original publication reporting the association of *NT5E* mutations and arterial calcification in patients with ACDC postulated increased ratio of P_i/PP_i as a trigger of ectopic mineralization as a result of increased TNAP activity.[8,11] This postulate was based on *in vitro* analysis of fibroblast cultures established from patients in a family with ACDC in comparison to the controls. However, the patients' serum phosphate levels and alkaline phosphatase activity were all within normal range.[8] In the *Nt5e*^{−/−} mice, the serum P_i levels were significantly increased and the plasma PP_i levels were markedly decreased leading to over 2-fold increase in the P_i/PP_i ratio. These observations are consistent with the postulate that in CD73 deficiency, there is an accelerated conversion of PP_i to P_i creating a pro-mineralization environment.

Attesting to the role of adenosine in progressive ectopic mineralization of skeletal tissues is a recent study reporting on mice lacking equilibrative nucleoside transporter 1 (ENT1), which transfers nucleosides, such as adenosine, across the plasma membrane.[30] This mouse was developed as a model for diffuse idiopathic skeletal hyperostosis (DISH), a non-inflammatory spondyloarthropathy characterized by ectopic mineralization of spinal tissues. This mouse model was generated through targeted deletion of exons 2–4 of the gene encoding ENT1. Micro CT of the homozygous mice revealed ectopic mineralization of perispinal tissues in the cervical-thoracic region and the knock out mice revealed large, irregular accumulation of eosinophilic material in

perispinal ligaments and entheses, intervertebral discs, and sternocostal articulations – some of these findings are reminiscent of those in CD73 deficient mice. However, similar to our findings on $Nt5e^{-/-}$ mice, the $Ent1^{-/-}$ mice showed no evidence of mineralization in blood vessels, indicating specificity of the mineralization process in these mice for the axial skeleton.[30] Collectively, the mouse models of ectopic mineralization, including those with CD73 and ENT1 deficiency, attest to the presence of a complex pro-mineralization/anti-mineralization network that under physiologic homeostatic conditions prevents ectopic mineralization of soft connective tissues.[12] Alterations in the components of this network, such as altered ratio of P_i/PP_i and the adenosine concentration, can result in mineralization pathways with phenotypic tissue specificity.

Materials and Methods

Mice and diet

The CD73 deficient mouse was developed by targeted disruption of the $Nt5e$ gene as previously described.[5] Wild-type, heterozygous and homozygous mice with respect to the mutant $Nt5e$ allele were generated from heterozygous matings initially on the 129SvEv strain background. Genotyping of $Nt5e$ gene was performed on genomic DNA isolated from tail clips and analyzed using PCR amplification. All the mice were maintained on an 'acceleration diet' (Harlan Teklad, Rodent diet TD.00442, Madison, WI, USA), which is enriched in phosphorus and has reduced magnesium content, compared with standard rodent diet (Laboratory Autoclavable Rodent Diet 5010: PMI Nutritional International, Brentwood, MO, USA).

Due to a homozygous $Abcc6$ mutation (rs32756904) in the 129SvEv strain background, the CD73 deficient mice were backcrossed with C57BL/6J mice for 3 times to replace this mutation in the $Abcc6$ gene with the wild type allele. Validation of the absence of the $Abcc6$ mutation in the progeny was performed by sequencing. Wild-type, heterozygous and homozygous mice with respect to the mutant $Nt5e$ allele were generated from heterozygous matings on the C57BL/6J strain background. All the mice were maintained on the "acceleration diet" as described above.

At 11–16 months of age, the mice were euthanized by CO_2 asphyxiation and necropsied for further analysis.[19] Mice were maintained under standard conditions at the Animal Facility of Thomas Jefferson University. All protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. Proper handling and care were practiced according to the animal welfare policies of the Public Health Service.

Small-animal CT (micro CT) scan

$Nt5e$ wild-type, heterozygous and homozygous mice were examined for mineralization at 11–16 months of age by small animal micro CT scan. Briefly, mice were anesthetized with a xylazine-ketamineacetopromazine cocktail (160 μl per 25 g body weight of 10 mg/kg xylazine, 200 mg/kg ketamine, 2 mg/kg acetopromazine) and then scanned with a MicroCAT II

(ImTek Inc., Oak Ridge, TN). A 3-dimensional rendering was created for each mouse using Amira software, version 3.1 (Visualization Sciences Group, Burlington, MA, USA).

Complete necropsy

After euthanizing the mice, complete necropsies were performed on a subset of 5 mice at the age of 12 months.[19] Briefly, tissues from all organs (Swiss rolls of the duodenum, jejunum, ileum, and colon (with anus and perineal skin), longitudinal section of the stomach with esophagus and cecum (inflated with fixative), cross sections of the left lateral and medial lobes of liver to include the gall bladder, spleen, left and right kidneys with adrenal glands, reproductive organs (testis, epididimis, accessory sex organs, male; ovary, uterine tube, uterus, mammary glands, female), preputial gland for males/clitoral gland for females, salivary gland cluster with cervical lymph nodes, heart, esophagus and trachea with thyroid and parathyroid glands, tongue, longitudinal sections out of the center of the lobes of both lungs, dorsal skin, ear skin (pinna), ventral skin, muzzle skin, eyelid, longitudinal section of hind leg, include stifle/knee joint, longitudinal section of front leg, include shoulder and elbow joints, longitudinal section of hind foot (soft tissues, bone, and nail unit/footpad), longitudinal section of front foot (soft tissues, bone, and nail unit/footpad), longitudinal section and 1 cross section of the lumbar spine, longitudinal section and 1 cross section of the tail, sections of the lower jaw) were collected and fixed in 10% phosphate-buffered formalin overnight, after which they were transferred and stored in 70% ethanol. Bones were processed in Cal-Ex® (Fisher Thermo Scientific, Waltham, MA, USA). Tissues were then trimmed and embedded in paraffin, cut into 6 μm sections, and stained with Hematoxylin-Eosin (H&E). All tissue slides were reviewed by an experienced, board-certified veterinary pathologist (JPS).

Histopathological analysis of mineralization

Organs from euthanized mice were fixed in 10% phosphate-buffered formalin and embedded in paraffin. The tissues were sectioned (6 μm thick), placed onto glass slides, and stained with Hematoxylin-Eosin (HE) or Alizarin Red (AR) stain using standard procedures. Slides were examined for mineralization under a Nikon (Tokyo, Japan) Te2000 microscope and an AutoQuant imaging system (AutoQuant Imaging, Watervliet, NY, USA).

Chemical quantification of calcium and phosphate in serum

Calcium levels in serum were determined colorimetrically by the o-cresolphthalein complexone method (Calcium (CPC) Liquicolor; Stanbio Laboratory, Boerne, TX, USA). Phosphate content in serum was determined with a Malachite Green phosphate assay kit (BioAssay Systems, Hayward, CA, USA).

Plasma collection and inorganic pyrophosphate assay

Whole blood was collected by cardiac puncture into heparin coated blood collection tubes and kept on ice until separation of plasma and erythrocytes by centrifugation. The plasma was collected, depleted of platelets by filtration ($2,200 \times$ g at $4°C$ for

20 min) through a Centrisart I 300,000-kDa mass cutoff filter (Sartorius, New York, NY, USA), and stored at −80°C until further processing. PPi in plasma was measured by an enzymatic assay using uridine-diphosphoglucose (UDPG) pyrophosphorylase, with modifications, as described previously.[31-33]

Alkaline phosphatase activity in serum

Serum levels of alkaline phosphatase were measured using a quantitative colorimetric assay kit from Abcam (Cambridge, MA, USA).

Statistical analysis

The comparisons between different groups of mice were completed using the 2-sided Kruskal–Wallis nonparametric test, comparable to one-way analysis of variance but without the parametric assumptions. All statistical computations were completed using the SPSS version 5.0 software (SPSS, Chicago, IL, USA).

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